Alterations in Sexually Dimorphic Biotransformation of Testosterone in Juvenile American Alligators (*Alligator mississippiensis*) from Contaminated Lakes

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The goal of this study was to determine whether hepatic biotransformation of testosterone is normally sexually dimorphic in juvenile alligators and whether living in a contaminated environment affects hepatic dimorphism. Lake Woodruff served as our reference site. Moonshine Bay, located on the west side of Lake Okeechobee, served as an intermediate site. Lake Apopka, the Belle Glade area located at the south end of Lake Okeechobee, and Water Conservation Area 3A, in the northern Everglades, served as our contaminated sites (all lakes are in Florida). Normal testosterone hydroxylase activity exhibited sexually dimorphic patterns of expression, with reference animals from Lake Woodruff exhibiting a female:male ratio of 1.44. This pattern was perturbed in all of the intermediate and contaminated sites investigated. Normal testosterone oxido-reductase activity exhibited sexually dimorphic expression (Lake Woodruff female:male ratio of 1.45). This pattern was altered in all contaminated sites investigated. UDP-Glucuronosyltransferase activity exhibited no sexually dimorphic pattern in animals collected from our reference site, with Lake Woodruff animals exhibiting a female:male ratio of 1.06. This pattern was perturbed in animals from Water Conservation Area 3A, which exhibited a female:male ratio of 0.65. Sulfotransferase activity demonstrated no sexual dimorphism at any of the sites investigated, although elevated activity was observed in males from the Lake Okeechobee watershed compared to those from Lake Woodruff. These data demonstrate different patterns of hepatic androgen biotransformation in animals living in contaminated environments. *Key words*: **alligator, hydroxylase, oxido-reductase, sexual dimorphism, sulfotransferase, testosterone biotransformation, UDP-glucuronosyltransferase.** *Environ Health Perspect* **109:1257–1264 (2001). [Online 28 November 2001]** *http://ehpnet1.niehs.nih.gov/docs/2001/109p1257-1264gunderson/abstract.html*

The liver plays an important role in maintaining homeostasis in all vertebrates. In addition to metabolizing toxicants, the liver plays an essential role in hormone homeostasis, as it metabolizes both peptide and steroid hormones. The hepatic metabolism of many drugs and steroids occurs in a sexually dimorphic manner (*1–5*) and thus could potentially serve as a biomarker for exposure to both naturally occurring and synthetic hormones. Maintenance of steroid homeostasis involves several variables including rate of hormone synthesis, interactions among hormones, rates of secretion, transport, biotransformation, and elimination (*6,7*). Homeostasis could be disrupted by xenobiotic-induced alterations of sex steroid metabolizing cytochrome P450 enzymes. By inducing or blocking these enzymes (both in the liver and in other tissues), the natural balance of circulating sex steroids could conceivably be altered from normal (*5,8,9*).

The liver has several mechanisms for hormone biotransformation. Direct conjugation, in which the steroid is conjugated to glucuronic acid or sulfate, produces a more water-soluble product that can then be excreted in bile and urine (*7,10*). Steroid hydroxylation accomplishes the same goal by stereo-selectively and regio-specifically attaching hydroxyl groups to the steroid (*7,11*), which also provides sites for subsequent conjugation reactions. Sex-specific differences in steroid hydroxylation, uridine diphosphate (UDP)-glucuronosyltransferase, and sulfotransferase enzymes have been investigated and well documented in mammals, birds, and reptiles, although gender patterns can vary among species (*2,3,10, 12–19*). Oxido-reduction of testosterone to androstenedione, dihydrotestosterone, and/or androstanediols is another hepatic biotransformation pathway that influences circulating levels of testosterone.

Sexually dimorphic expression of sex steroid-metabolizing enzymes has been well documented. Rats exhibit considerable sexual dimorphism in hepatic biotransformation, which has been linked to the pulsatile secretions of growth hormone from the pituitary, under feedback control from estrogens and testosterone (hypothalamo–pituitary–hepatic axis) (*12,13,15*). This sexual dimorphism is imprinted during development, a process mediated by sex steroids (*13*). Fish, birds, and marine mammals are among the other groups that have demonstrated sexual dimorphism in cytochrome P450 enzymes (*3,9,17,20*). Glucuronosyltransferase activity is responsible for conjugating xenobiotics and endogenous substrates such as bilirubin, catecholamines, and steroids and has been shown in all mammals, birds, and reptiles investigated. Sex differences appear to be hormonally related and imprinted during development (*10*). Vertebrate sulfotransferases exhibit species and sex-specific differences (androgen dependent in some cases) and are responsible for the esterification of steroids, carbohydrates, proteins, and xenobiotics (*10,19*).

A disruption in the expression of sexually dimorphic enzymes by endocrine-altering compounds could provide an explanation for the skewed sex steroid concentrations observed in alligators collected from Lake Apopka, a highly contaminated lake, when compared to Lake Woodruff, a relatively pristine wildlife sanctuary in central Florida (*21*). Lake Apopka alligators exposed to chemicals from normal agricultural activity, municipal runoff, and the 1980 Tower Chemical Company spill [containing dicofol, DDT, and DDE (*22*)] had skewed sex hormone ratios (estrogen/testosterone), and males exhibited altered plasma testosterone levels when compared to Lake Woodruff (*23*). Males from Lake Apopka had depressed plasma testosterone concentrations that were comparable to female plasma testosterone concentrations; these concentrations were three times lower than those in Lake Woodruff males (reference animals). There is considerable evidence in the literature that demonstrates hormonal activity by common anthropogenic compounds in the environment (*24–26*). Possible mechanisms through which normal endocrine homeostasis could be disrupted by hormonally active agents

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We thank J.B. McLachlan and C. Ryder for help with the biotransformation assays, and A. Woodward, M.R. Milnes, S. Kools, S. Degala, J. Gates, and the Florida Fish and Wildlife Conservation Commission for the invaluable help they offered in the collection of the animals.

Funding for this project was provided by U.S. EPA grants (CR 826357-01-1 and CR 162460-91-6).

Fieldwork was conducted under permit from the Florida Fish and Wildlife Conservation Commission. All laboratory work was conducted in compliance with the guidelines of the University of Florida Institutional Animal Care and Use Committee.

Received 16 January 2001; accepted 8 May 2001.

include, altering *a*) the hypothalamic–pituitary axis; *b*) the activity of steroidogenic enzymes (developmentally or directly); *c*) the function of steroid binding molecules such as sex hormone-binding globulin; *d*) the activity of hormone receptors by acting as hormone agonists or antagonists; *e*) the hepatic rate of steroid inactivation; or *f*) renal clearance (*6*). The pattern of *in vitro* steroidogenic activity of the gonads collected from Lake Apopka and Lake Woodruff animals did not match the circulating plasma concentrations of testosterone observed in the same individuals (*23,27*). Similar rates of testosterone synthesis were observed *in vitro* when testes obtained from animals from each lake were compared (*27*); this was in contrast to differences observed in circulating plasma concentrations (*23*). Guillette et al. (*27*) hypothesized that the differences in sex steroid concentrations seen between *in vivo* and *in vitro* patterns could be due, in part, to modifications in hepatic biotransformation.

In the present study, we evaluated hepatic testosterone hydroxylase, sulfotransferase, UDP-glucuronosyltransferase, and oxidoreductase activities for sexual dimorphism that could be regulated by androgens and estrogens in alligators. We investigated the status of these dimorphisms in juvenile alligators collected from one established reference site, one site considered to be intermediately contaminated, and three contaminated sites. We hypothesized that, like rodents (*12–15*) and chickens (*3*), sexually dimorphic patterns of testosterone metabolism would be observed in juvenile American alligators from the relatively pristine reference site, Lake Woodruff National Wildlife Refuge. Secondly, we hypothesized that altered hepatic enzyme activity would be observed in juvenile American alligators collected from intermediate (Moonshine Bay) and contaminated sites [Lake Apopka, the Belle Glade area on Lake Okeechobee, and Water Conservation Area 3A (WCA 3A) in the northern Everglades] when compared to our reference sites.

Materials and Methods

Study sites. Lake Woodruff (Figure 1) is located within a relatively pristine national wildlife sanctuary and has no known history of point source pollution from agricultural or industrial activity and has minimal development. It has served as a reference site for our laboratory throughout the last decade (*21,23,27,28*). Animals from this lake have demonstrated consistently higher plasma testosterone levels, larger phallus sizes, lower levels of organochlorine compounds, and higher hatching rates than other lakes investigated, including Lake Apopka (*21,23,28*).

In contrast, Lake Apopka (Figure 1) has had a confirmed industrial spill containing organochlorine compounds (*22*) and serves as a catch basin for numerous chemicals historically used in muck farming, as well as chemicals used in lawn care products from housing developments and towns that have grown around the lake in recent years. Juvenile alligators from this lake exhibit elevated serum concentrations of *p,p′*-DDE, dieldrin, endrin, mirex, oxychlordane, a combination of DDTs, and a combination of polychlorinated biphenyls (PCBs) when compared to juveniles from the other lakes, including Lake Woodruff (*28*). These patterns are not unique to Lake Apopka and Lake Woodruff and have recently been demonstrated on other lakes in Florida (*21*).

Lake Okeechobee (Figure 1) and its tributaries have been the catch basins of agricultural runoff containing pesticides and animal wastes (*29,30*). Historically, Moonshine Bay, our intermediate site on Lake Okeechobee, has shown detectable sediment levels of DDE (1.2 µg/kg) and dichlorodiphenyldichloroethane (DDD; 2.4 µg/kg) that are lower than those reported for the Belle Glade area on the south shore of Lake Okeechobee and WCA 3A in the northern Everglades (*30*). More recent sampling at the mouth of Fish Eating Creek (located near Moonshine Bay) has shown DDD and DDE to be nondetectable in sediments (*31,32*). The south end of the lake (Belle Glade area) has three islands, Kreamer Island, Torry Island, and Rita Island. Torry and Rita Islands have had documented organochlorine levels (DDT, DDD, DDE) ranging from 2,200 to 110,000 µg/kg sediment (*30*). This is not surprising due to the agricultural activities (muck farming) that historically occurred on these islands. More recent reports show that sediment samples collected from the south end of the lake contain detectable levels of DDD (ranging from near detection limits to 24 µg/kg) and DDE (7.4–47 µg/kg) (*31,32*). WCA 3A has had reported sediment levels of DDE ranging from 3.8 to 150 µg/kg and DDD ranging from 0.1 to 62 µg/kg (*30*). Chlordane, DDT, dieldrin, and endrin were all reported at levels < 0.1 µg/kg (*30*). More recent sampling in and around WCA 3A has shown DDD levels at or slightly above the minimum detection limit, whereas DDE levels range from slightly above the minimum detection limit to 21 µg/kg sediment (*31,32*). Altered circulating testosterone concentrations have recently been detected in juvenile alligators from Lake Okeechobee (*21,33*).

Animal and tissue collection. Juvenile alligators (0.9–1.5 m) were caught by hand from each of the five sites studied. Animals were collected from Lake Woodruff (n = 5 males, n = 4 females) and Lake Apopka (n = 5 males, n = 4 females) during the early summer (June) of 1997. Animals were collected from Moonshine Bay (n = 5 males, n = 5 females), Belle Glade (n = 7 males, n = 4 females), and WCA 3A (n = 5 males, n = 4 females) on consecutive nights in May 1999. We recorded plasma samples, body weights, snout–vent lengths, and total lengths for each animal. Animals were then transported to the River Woods Field Station (run by the South Florida Water Management District) located near Lake Okeechobee where necropsies were conducted. Necropsies were performed within 5–12 hr after capture. Animals were euthanized via a lethal dose of sodium pentobarbitol injected into the spinal vein. Liver tissue was removed immediately, sliced into 2 g pieces, and flash frozen in liquid nitrogen. Samples were stored at –70°C until microsomes were prepared for assay.

Although sodium pentobarbital is a cytochrome P450 inducer, Ertl et al. (*34*) recently demonstrated that no significant induction of cytochromes P450 activities is apparent in alligators until 48 hr after injection of pentobarbital, with basal or less than basal levels of activity being measured before 24 hr postexposure. We therefore do not believe that the pentobarbital solution used to euthanize these animals affected the results because we removed the tissues immediately after injecting the alligators with pentobarbital.

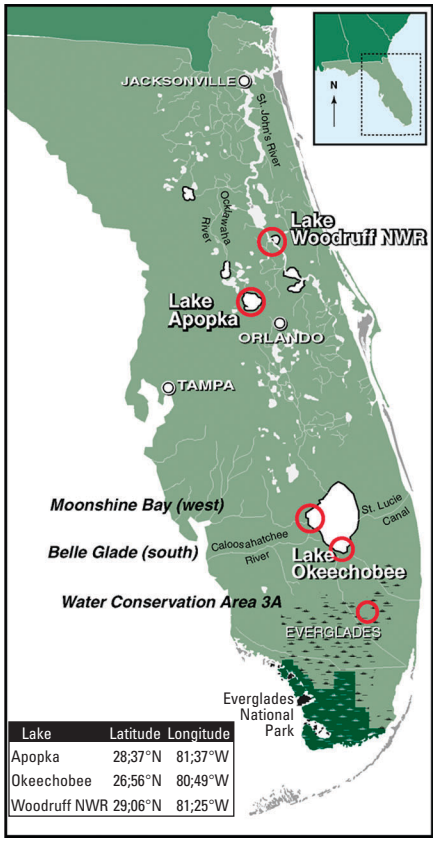

Figure 1. Map of Florida showing sites investigated in this study.

Cytosol and microsome preparation from alligator livers. We isolated microsomes and cytosol from juvenile alligator livers using the techniques outlined by Baldwin and LeBlanc (*35*) and Wilson and LeBlanc (*7*). For each animal, 2 g liver tissue was thawed and homogenized in ice-cold cytosol buffer [0.01 M HEPES (pH 7.4), 1 mM EDTA, 10% glycerol] with a Dounce homogenizer (Kontes, Vineland, NJ). Homogenate was first centrifuged at 9,000 × *g* for 15 min. The supernatant was poured off and centrifuged at 100,000 × *g* for 1 hr. The supernatant (cytosol) was poured off, aliquoted, and stored at –80°C for sulfotransferase activity. The pellet (microsomes) was resuspended in microsome buffer [0.1 M potassium phosphate buffer (pH 7.4), 1mM EDTA, 20% glycerol] and centrifuged again at 100,000 × *g* for 1 hr. The supernatant was poured off and the pellet was resuspended in microsome buffer, aliquoted, and stored at –80°C. Protein concentrations were determined using Bradford Reagent (Biorad, Hercules, CA) with bovine serum albumin (Sigma, St. Louis, MO) as the standard (*35,36*).

Testosterone hydroxylase and oxido-reductase activity. [^{14}C]Testosterone (40 nmol) in toluene was dried down in tubes under a steady stream of nitrogen for 5 min, as previously described (*7,35*). We then added potassium phosphate buffer (0.1 M, pH 7.4) and microsomal protein (400 µg protein) to each tube containing [^{14}C]testosterone to a total volume of 375 µL. Tubes were incubated in a shaking water bath (37°C) for 10–15 min. NADPH (25 µL at 14.3 mg/mL) was then added to each sample to initiate the reaction. The samples were incubated for 1 hr at 37°C. Product formation was linear over this period. The reaction was stopped by adding 1 mL ethyl acetate and vortexing briefly. The samples were then centrifuged at medium speed for 10 min to separate the ethyl acetate and aqueous phases. The ethyl acetate layer was removed and placed in a fresh tube. The aqueous layer was extracted two more times, first with 2 mL ethyl acetate and then with 1 ml ethyl acetate. We evaporated the ethyl acetate extract under a steady nitrogen stream. The samples were resuspended in 70 µL ethyl acetate (35 µL × 2) and spotted on aluminum backed silica thin-layer chromatography (TLC) plates; the metabolites were allowed to separate in a tank containing 80% methylene chloride/20% acetone and then in a tank containing 73% chloroform/18% ethyl acetate/9% ethanol. Unmetabolized [^{14}C]testosterone and [^{14}C]testosterone hydroxylated and oxido-reduced metabolites were quantified using a Packard Instant Imager (Packard Instrument Co., Downers Grove, IL) and compared to previously identified metabolites generated with mouse microsomes (*35*). We accounted for spontaneous hydroxylase and oxido-reductase activities by running a tube containing all the assay components except the microsomal protein, and we accounted for basal levels of activity by running a tube containing all of the assay components except NADPH. Specific activity for each metabolite was calculated as picomoles of metabolite per minute per milligram of microsomal protein. Total oxido-reduction metabolite formation was calculated as the sum of the metabolites that comigrated with the mouse dihydrotesterone, androstenedione, and androstanediols. Hydroxylated metabolite formation was calculated as the sum of the metabolites that comigrated in the same region as hydroxylated metabolite standards (*35*). The activities associated with the two blanks (either in the absence of NADPH (basal) or microsomal protein) were averaged and subtracted from the activities associated with each metabolite. Basal activities were 36.7 pmol/min/mg protein for total hydroxylase activity and 74.8 pmol/min/mg protein for total oxido-reductase activity.

Testosterone UDP-glucuronosyltransferase activity. UDP-glucuronosyltransferase activity was assayed as described previously (*7*). Briefly, we incubated 200 µg microsomal protein with [^{14}C]testosterone (40 nmol; 1.8 µCi/mmol) in 0.1M potassium phosphate buffer, pH 7.4 (total reaction volume 400 µL). We initiated the reaction by adding 0.129 mg uridine 5′-diphosphoglucuronic acid and allowed it to incubate at 37°C for 1 hr. Product formation was shown to be linear over this period. We stopped the reaction by adding 2 mL of ethyl acetate and then vortexing thoroughly. The samples were then centrifuged for 10 min at medium speed. The ethyl acetate layer was removed and the aqueous layer extracted a second time with 2 mL ethyl acetate. The samples were centrifuged a second time for 10 min. The aqueous layer (100 µL) was then removed and added to 5 mL of scintillation cocktail for counting using a scintillation counter. We accounted for spontaneous glucuronic acid conjugation to testosterone by running tubes that had all the assay components except the microsomal protein. We measured basal glucuronic acid conjugation to testosterone by including a tube containing all of the assay components except uridine 5′-diphosphoglucuronic acid. Basal glucuronic acid conjugation to testosterone was 0.96 pmol/min/mg protein. The activities associated with the two blank tubes (basal activity or spontaneous glucuronic acid conjugation) were averaged, and this amount was subtracted from the calculated specific activities. UDP-Glucuronosyltransferase activity was calculated as picomoles per minute per milligram of microsomal protein (*7*).

Sulfotransferase activity. We measured sulfotransferase activity using techniques described previously (*7*). Briefly, we incubated 200 mg cytosolic protein with [^{14}C]testosterone (40 nmol; 1.8 µCi/mmol) in 0.1M potassium phosphate buffer, pH 6.0 (total reaction volume 400 µL). The reaction was initiated by adding 10 µL of PAPS (adenosine 3′ phosphate-5′-phosphosulfate at 10.1 mg/mL) and allowed to incubate at 37°C for 1 hr. Product formation was shown to be linear over this period. The reaction was stopped by adding 2 ml ethyl acetate and vortexing thoroughly. The samples were then centrifuged for 10 min at medium speed. The ethyl acetate layer was removed and the aqueous layer extracted a second time with 2 mL ethyl acetate. The samples were centrifuged a second time for 10 min. The aqueous layer (100 µL) was then removed and added to 5 ml scintillation cocktail for counting using a scintillation counter. Product formation was quantified by measuring the amount of radioactivity present in the 100 µL aqueous sample after it had been extracted twice with ethyl acetate. Sulfate-conjugated products extracted into the ethyl acetate were separated from the [^{14}C]testosterone using TLC (80% methylene chloride/20% acetone), the origin was cut out, and the radioactivity was measured using 5 mL scintillation fluid and a scintillation counter. This amount was added to the calculated specific activities. We accounted for spontaneous sulfate conjugation to testosterone by running tubes containing all the assay components except cytosolic protein. Basal sulfate conjugation to testosterone was measured by including a tube containing all the assay components except PAPS. Basal sulfate conjugation to testosterone was 0.15 pmol/min/mg protein. The activities associated with the two blank tubes (basal activity or spontaneous sulfate conjugation) were averaged, and this amount was subtracted from the calculated specific activities. Sulfotransferase activity was calculated as picomoles per minute per milligram of microsomal protein (*7*).

Statistical analysis. Significant differences ($p \leq 0.05$) in snout–vent length within and among the sites were shown using one-way analysis of variance (ANOVA). To determine whether hepatic enzyme activity covaries with body size in juvenile alligators, we plotted snout–vent length against UDP-glucuronosyltransferase activity ($n = 48$) and demonstrated a significant negative regression ($p = 0.0001$). Statistical significance ($p < 0.05$) was determined using analysis of covariance (ANCOVA; with snout–vent length as the covariate) to compare male and female activities within each site. ANCOVA

and Fisher's protected least significant difference (PLSD) post hoc analysis was used when males and females were compared between sites. All statistical analyses were performed using Statview (SAS Institute, Cary, NC). Female:male ratios were calculated using the mean activity values for females and males from each site.

Results

Alligator microsomes biotransformed testosterone to 15 quantifiable metabolites using the current methodology. We assigned identities to these metabolites based on the mouse metabolites that comigrated with these metabolites on TLC plates. Three of the metabolites comigrated with androstenedione, dihydrotestosterone, and androstanediol. We grouped these metabolites together as oxido-reduction metabolites. Nine metabolites migrated in the region that is generally associated with monohydroxylated derivatives of testosterone (A1-OH to A9-OH). Seven of these metabolites comigrated with 2α-OH (A1-OH), 16β-OH (A2-OH), 6β-OH (A3-OH), 6α-OH (A4-OH), 15β- and 7α-OH (A7-OH), 16α-OH (A8-OH), and 15α-OH (A9-OH). For the hydroxylase metabolites, the results varied negligibly whether we included just the seven metabolites that comigrated with the known hydroxy derivatives or all of the apparent nine metabolites quantifiable from the alligator microsomes. For purposes of this analysis, we collectively refer to the nine alligator hydroxy metabolites as hydroxylase enzyme-derived metabolites. Glucuronic acid and sulfate-conjugated testosterone metabolites were also produced in alligator liver samples.

Testosterone hydroxylase activities. Sexually dimorphic patterns of testosterone hydroxylase activity were evident in our reference site, with females exhibiting higher activity than males (Table 1, Figure 2). Lake Woodruff animals exhibited a female:male ratio of 1.44 (p = 0.008). Animals from the intermediately contaminated site, Moonshine Bay, did not exhibit a sexually dimorphic pattern in testosterone hydroxylase activity (p = 0.64). Female:male ratios are expressed using the mean activities for males and females from each site.

The normal sexual dimorphism in testosterone hydroxylase activity, based on Lake Woodruff animals, was perturbed in all of the contaminated sites investigated (Table 1, Figure 2). The dimorphism was not apparent in WCA 3A animals (p = 0.11), Belle Glade animals (p = 0.056), or Lake Apopka animals (p = 0.36). Because of the small sample size, we combined all of the south Florida sites (Moonshine Bay, Belle Glade, and WCA 3A) since there is a history of varying degrees of contaminant exposure throughout the watershed. No sexual dimorphism was observed (female:male ratio 0.99; p = 0.9) among the pooled animals (Table 1, Figure 2). Testosterone hydroxylase activity was not significantly different (p = 0.16) among females when compared to Lake Woodruff females. Males from south Florida

Table 1. Summary of total testosterone hydroxylase and oxido-reduction activities (pmol/min/mg microsomal protein ± SE) in juvenile allegators from Lake Woodruff, Lake Apopka, Lake Okeechobee, and WCA 3A.

Site/sex	Hydroxylase	F:M ratio	Oxido-reduction	F:M ratio
Woodruff				
M	169.6 ± 17.2	*1.44	187.1 ± 23	*1.45
F	244.3 ± 9.3		271 ± 17.8	
Apopka				
M	215 ± 31.4	0.67	180.5 ± 18.4	0.98
F	143 ± 28.6		178 ± 7.3	
South Florida grouped				
M	226.8 ± 25.3	0.99	183.4 ± 13.9	1.13
F	223.6 ± 35.3		208.1 ± 13.4	
Moonshine Bay				
M	184.9 ± 35.7	1.59	193.9 ± 28.9	1.21
F	293.7 ± 100.1		234.3 ± 27.6	
Belle Glade				
M	200.9 ± 40.1	0.82	154.0 ± 22.2	1.47
F	165.2 ± 8.9		226.6 ± 12.3	
WCA 3A				
M	304.9 ± 43	0.69	214 ± 13.4	*0.76
F	211.7 ± 27.7		163 ± 7.7	

Abbreviations: F, female; M, male.
*Significant difference (p < 0.05) between males and females from that site.

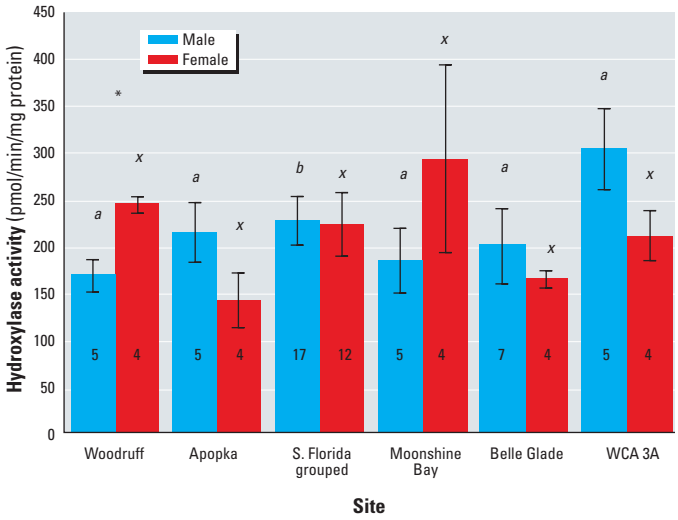

Figure 2. Total testosterone hydroxylase activity expressed as picomoles of metabolite per minute per milligram of microsomal protein. Values within bars denote sample size.
*Statistically significant difference (p < 0.05) between males and females at that site. Significant differences (p < 0.05) between sites, relative to Lake Woodruff, are indicated using *a* and *b* for male comparisons and *x* and *y* for female comparisons.

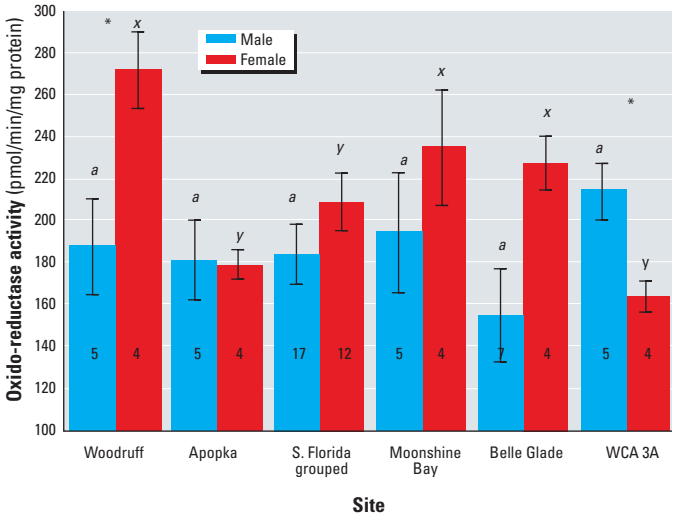

Figure 3. Total testosterone oxido-reductase activity expressed as picomoles of metabolite per minute per milligram of microsomal protein. Values within bars denote sample size.
*Statistically significant difference (p < 0.05) between males and females at that site. Significant differences (p < 0.05) between sites, relative to Lake Woodruff, are indicated using *a* and *b* for male comparisons and *x* and *y* for female comparisons.

(three sites grouped) exhibited elevated activity when compared to Lake Woodruff males ($p = 0.02$) (Table 1, Figure 2). Thus, testosterone hydroxylase activity is normally sexually dimorphic in juvenile alligators, and this dimorphism is altered in contaminated sites.

The variation observed in Moonshine Bay females is due to one of the four females (one sample was lost due to experimental error) having a total hydroxylase activity greater than 2 standard deviations from the mean. The female was kept in the data set due to the already small sample size. When that one female is removed, there is still no sexually dimorphic pattern in the animals from that site ($p = 0.98$), which exhibit a female:male ratio of 1.07. Small sample size is a problem commonly faced with protected or threatened species, where limited numbers of animals can be collected for necropsy. Our laboratory is presently working on noninvasive techniques to screen large numbers of animals from different populations.

Testosterone oxido-reductase activities. As observed for testosterone hydroxylase activity, normal oxido-reductase activity in juvenile alligators demonstrated sexually dimorphic patterns, with females having greater activity than males (Table 1, Figure 3). Lake Woodruff animals (reference) exhibited a female:male ratio of 1.45 ($p = 0.027$).

This normal sexual dimorphism in testosterone oxido-reductase activity was altered in animals from all of the other sites (Figure 3). WCA 3A animals exhibited a significantly reversed pattern ($p = 0.01$), with a female:male ratio of 0.76. The normal sexually dimorphic pattern did not exist in Moonshine Bay (intermediate) ($p = 0.68$), Belle Glade ($p = 0.16$), or Lake Apopka ($p = 0.38$) animals. When Moonshine Bay, Belle Glade, and WCA 3A animals were grouped together, no sexual dimorphism was observed ($p = 0.23$). The perturbation of the sexually dimorphic pattern observed in animals from Lake Apopka, WCA 3A, and south Florida grouped sites is due to a significant reduction ($p \leq 0.03$) in activity in females as compared to Lake Woodruff animals. Therefore, we conclude that total oxido-reductase activity is sexually dimorphic in juvenile alligators and that animals collected from contaminated sites exhibit altered patterns of activity. In some cases, these altered patterns are due to sex-specific decreases in activity.

Testosterone UDP-glucuronosyltransferase activity. UDP-glucuronosyltransferase activity was not sexually dimorphic in Lake Woodruff animals ($p = 0.67$) (Table 2, Figure 4). This same pattern was observed in our intermediate site, Moonshine Bay ($p = 0.78$).

The pattern of UDP-glucuronosyltransferase activity was significantly altered in one of the contaminated sites. WCA 3A ($p = 0.03$) exhibited sex-specific differences with a female:male ratio of 0.65 (Table 2). Belle Glade animals exhibited a nearly significant difference ($p = 0.056$), with a ratio of 0.77. Lake Apopka animals exhibited no sex-specific differences ($p = 0.65$). When Moonshine Bay, Belle Glade, and WCA 3A were grouped together, no sex-specific differences were observed ($p = 0.46$). No significant differences among males ($p = 0.65$) or females ($p = 0.18$) were observed when compared to Lake Woodruff animals. When South Florida grouped and Lake Apopka animals were compared to Lake Woodruff animals, again no differences were observed between males ($p = 0.91$) or females ($p = 0.12$). We conclude that UDP-glucuronosyltransferase activity is not normally sexually dimorphic in juvenile alligators and that, in some cases, animals living in contaminated sites exhibit alterations in this pattern.

To determine whether hepatic enzyme activity varied with body size in juvenile alligators, we grouped all of the animals from the five sites together and plotted UDP-glucuronosyltransferase activity against snout–vent length. A significant negative regression (inverse relationship)($p < 0.002$) was obtained for females ($n = 21$; $r^2 = 0.40$), males ($n = 27$; $r^2 = 0.62$), and males and females grouped together ($n = 48$; $r^2 = 0.54$), with activity decreasing with increased snout–vent length. We examined UDP-glucuronosyltransferase activity in this case because no sexual dimorphism was observed in the reference sites. We realize that the site differences reported could influence these findings, although significant regressions were observed for both sexes examined separately. Based on these results, we analyzed the different data sets examined in this study using ANCOVA using snout–vent length as the covariate.

Testosterone sulfotransferase activity. Sulfotransferase activity exhibited no sexual dimorphism in Lake Woodruff animals ($p = 0.14$) (Table 2, Figure 5). Animals collected from our intermediate and contaminated sites did not exhibit altered intersex patterns of sulfotransferase activity [Moonshine Bay animals (intermediate) ($p = 0.92$), Lake Apopka ($p = 0.82$), Belle Glade ($p = 0.62$), WCA 3A ($p = 0.16$)]. When Moonshine Bay, Belle Glade, and WCA 3A animals were grouped together, no sexual dimorphism was observed in sulfotransferase activity ($p = 0.64$). Males from the contaminated sites demonstrated differences, with males from Moonshine Bay ($p = 0.04$), Belle Glade ($p = 0.03$), WCA 3A ($p = 0.001$), and south Florida sites grouped together ($p = 0.005$) all exhibiting elevated sulfotransferase activity when compared to Lake Woodruff. We conclude that sulfotransferase activity is not normally sexually dimorphic in juvenile alligators. Males collected from the sites in south Florida exhibit elevated sulfotransferase activity, although this alteration is not pronounced enough to perturb the normal patterns observed between males and females.

Discussion

We designed this study to test two hypotheses. First, we hypothesized that sexually dimorphic hepatic testosterone biotransformation would be observed in animals collected from our relatively pristine reference site, Lake Woodruff. Second, we hypothesized that altered testosterone biotransformation would be observed in our experimental sites containing known and suspected levels of organochlorine pesticides among other chemicals (intermediate site: Moonshine Bay; contaminated sites: Lake Apopka, Belle Glade area, and WCA 3A) when compared to our reference site (*28,30*). DDT, its metabolites, and other organochlorine compounds have been shown to have multiple endocrine-disrupting effects on exposed organisms, ranging from binding the androgen receptor to altering steroid concentrations to regulating hepatic cytochrome P450 activity in a sex-dependent manner (*16,24,37*).

In this study, we observed sexually dimorphic total hydroxylase activity in juvenile alligators collected from Lake Woodruff, with higher activity being observed in females. These findings are interesting in light of the fact that sexual dimorphisms in hepatic hydroxylase enzymes have been linked to estrogen and androgen exposure in mice and rats. Drug-metabolizing enzymes appear to be androgen dependent in rats, which exhibit a 600% difference between males and females (*3*). In contrast, mice show a pattern in which females have greater drug-metabolizing activity than males (30–100%); androgen has a repressive effect on enzyme activity [reviewed by Pampori and Shapiro (*3*)]. Goats exhibit a pattern similar to mice, whereas chickens have sexually dimorphic drug-metabolizing patterns closer to those observed in rats (*3*). Our data from alligators obtained from Lake Woodruff indicate that females exhibit greater testosterone metabolism than males. This pattern is similar to that reported for mice and goats, where testosterone appears to suppress the enzyme systems responsible for its biotransformation.

The sexually dimorphic testosterone hydroxylase pattern observed in Lake Woodruff animals was not exhibited in Moonshine Bay area animals. This suggests that this site is not a "pristine" population when compared to Lake Woodruff and that it is an intermediate site between Lake Woodruff and Belle Glade, given the fact that both of these sites (Woodruff and Belle Glade) exhibit significant or near significant

(p = 0.056 in the case of Belle Glade) sexual dimorphism, but with opposite patterns. Further, it could be argued that Lake Apopka, Moonshine Bay, and WCA 3A are all intermediate between Lake Woodruff and Belle Glade (essentially best and worst case scenarios) because none of these sites exhibited sexual dimorphisms. We realize that the difference between the sexes observed in Belle Glade alligators (p = 0.056) is not significant at the 0.05 level, but we feel it is strongly suggestive given our relatively small sample size.

Testosterone hydroxylase activity was altered in all of the contaminated sites investigated. Serum testosterone concentrations are altered not only in Lake Apopka alligators but also in other lakes in Florida, including Lake Okeechobee (*21*). Further work is needed on hepatic inactivation of testosterone through hydroxylation pathways in alligators to explore whether xenobiotic exposure could disrupt these enzymes that help maintain serum levels of testosterone. Most of the work on vertebrates thus far has been done on rodent models. Compounds such as ketoconozole, indole-3-carbinol, endosulfan, and DDT, to name a few, differentially modulate hepatic enzyme activity with varying effects on serum testosterone concentrations (*5,7,8,16*). The 6α/15α hydroxylase ratio (masculinized less than feminized) has been proposed as an indicator of the androgen status of the animal based on the fact that these enzymes are influenced by the presence or absence of androgen, although its effectiveness depends on the compound to which the animal was exposed (*5,8*). Wilson and LeBlanc (*8*) recently presented data suggesting that ketoconazole differentially modulates hepatic testosterone biotransformation activities in mice but that the lowered plasma testosterone concentrations are due to decreased gonadal synthesis. Additional studies that investigate the impacts of *in vitro* culture of gonads in the presence of various contaminants would certainly provide valuable information concerning the altered plasma concentrations of hormones in alligators living in contaminated environments. Furthermore, work needs to be done to characterize the specific cytochrome P450 enzymes driving the differences observed in alligators in this study to determine if the observed differences can be related to cytochromes P450 previously described in alligators (*34,38,39*).

Normal testosterone oxido-reductase activity was sexually dimorphic in Lake Woodruff animals, with females exhibiting higher activity than males. It is of interest that adult female fathead minnows (*Pimephales promelas*) eliminate androstanediol at a higher rate than males (*9*), matching the pattern we observed in total oxido-reduction metabolite formation in Lake Woodruff animals. Testosterone elimination pathways in crocodilians should be investigated further to determine if oxido-reductase metabolites are a major elimination product or whether they serve primarily as a precursor pool. Animals

Table 2. Summary of total testosterone UDP-glucuronosyltransferase and sulfotransferase activities (pmol/min/mg microsomal protein ± SE) in juvenile alligators from Lake Woodruff, Lake Apopka, Lake Okeechobee, and WCA 3A.

Site/sex	UDP-Glucuronosyltransferase	F:M ratio	Sulfotransferase	F:M ratio
Woodruff				
M	45.2 ± 8.42	1.06	1.9 ± 0.1	1.21
F	47.8 ± 5.4		2.3 ± 0.2	
Apopka				
M	50.47 ± 4.32	0.59	2.54 ± 0.44	0.97
F	29.89 ± 3.74		2.46 ± 0.3	
South Florida grouped				
M	31.9 ± 2.7	0.89	2.8 ± 0.1	0.96
F	28.5 ± 3.6		2.7 ± 0.1	
Moonshine Bay				
M	24.5 ± 2.3	1.38	2.6 ± 0.2	1.04
F	33.8 ± 8.8		2.7 ± 0.3	
Belle Glade				
M	29.3 ± 4.1	0.77	2.6 ± 0.1	0.96
F	22.5 ± 0.76		2.5 ± 0.2	
WCA 3A				
M	43 ± 3.5	*0.65	3.1 ± 0.2	0.87
F	27.9 ± 3.6		2.7 ± 0.2	

Abbreviations: F, female; M, male.
*Significant difference (p < 0.05) between males and females from that site.

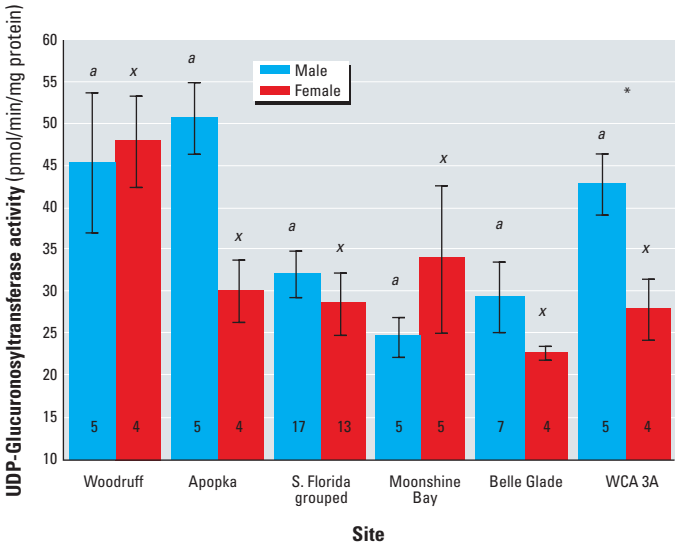

Figure 4. UDP-Glucuronosyltransferase activity in male and female juvenile alligators expressed as picomoles of metabolite per minute per milligram of microsomal protein. Values within bars denote sample size.
*Statistically significant difference (p< 0.05) between males and females at that site. Significant differences (p < 0.05) between sites, relative to Lake Woodruff, are indicated using *a* and *b* for male comparisons and *x* and *y* for female comparisons.

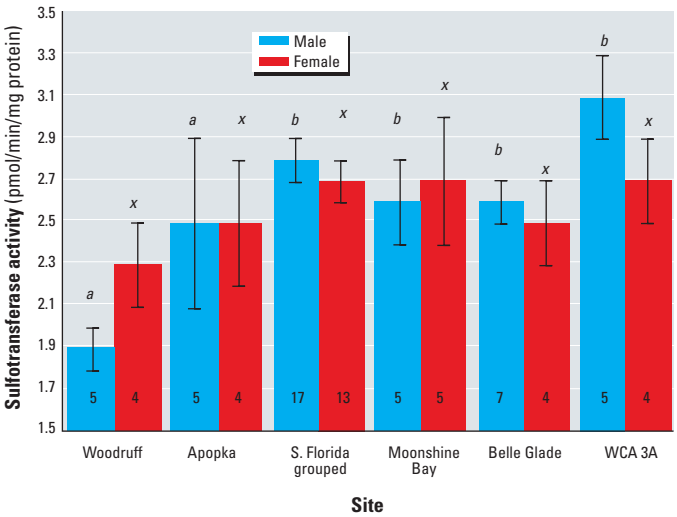

Figure 5. Testosterone sulfotransferase activity in male and female juvenile alligators expressed as picomoles of metabolite per minute per milligram of microsomal protein. Values within bars denote sample size.
*Statistically significant difference (p < 0.05) between males and females at that site. Significant differences (p < 0.05) between sites, relative to Lake Woodruff, are indicated using *a* and *b* for male comparisons and *x* and *y* for female comparisons.

from Moonshine Bay exhibited high variation in oxido-reductase activity; thus, conclusions are difficult with regard to differences between the sexes. Excessive variation (as seen in this study) due to small sample sizes is a problem that is commonly encountered when studying protected species, where it is neither desirable nor feasible to kill large numbers of animals. We attempted to deal with this problem by combining all of the animals collected from south Florida (Moonshine Bay, Belle Glade, and Water Conservation area 3A) for comparison with Lake Woodruff, even though these sites are not identical in terms of the reported sediment contaminant levels. Our laboratory is presently developing noninvasive techniques to investigate steroid metabolite formation in alligators that will enable the sampling of large numbers of animals from different populations.

Oxido-reductase patterns exhibited in animals from all of the contaminated sites investigated were different from those observed in Lake Woodruff animals. As we suggested for the testosterone hydroxylase enzymes, Moonshine Bay, Belle Glade, and Lake Apopka apparently represent intermediate exposure sites between Lake Woodruff and, in this case, WCA 3A, both of which exhibited sexually dimorphic profiles but with opposite female:male ratio patterns, 1.45 and 0.76, respectively. Given the fact that we are investigating a different suite of enzymes that could be acted on by different compounds, WCA 3A animals could be the most susceptible or exposed population examined. Lake Apopka, Lake Okeechobee, and WCA 3A could have different mixtures of compounds present, thus compounding the complexity. In-depth contaminant analysis of the five sites would offer valuable information for this study.

Juvenile alligators from the reference site did not exhibit sexually dimorphic patterns of UDP-glucuronosyltransferase activity. In contrast, males from two of the contaminated sites (WCA 3A and Belle Glade) exhibited a significant or near significant ($p = 0.056$ for Belle Glade) sexually dimorphic pattern, with males demonstrating higher activities than females. Compounds found in fungicides and pulp mill effluents have been shown to modulate UDP-glucuronosyltransferase activity in rodent and fish models (*40,41*). The relative sex-specific differences in UDP-glucuronosyltransferase activity observed in animals collected from Belle Glade and WCA 3A warrant further investigation, especially because glucuronidation is an important clearance pathway for many modern pesticides (*10*).

Sulfotransferase activity normally exhibits no sexual dimorphism in expression in juvenile alligators. Significant elevations in activity were observed, however, in males from the Lake Okeechobee watershed compared to those from Lake Woodruff. These differences did not alter the balance between males and females enough to exhibit a significant difference between the sexes.

It has been demonstrated in rodents that the hypothalamo–pituitary–liver axis is influenced by hormonal signaling during development (*12,13,15,30*). It is therefore a logical suggestion that this system is potentially susceptible to alteration by hormonally active agents in the environment. Lake Apopka and Lake Okeechobee both have documented histories of exposure to such compounds through municipal and agricultural run off (*22,29–32,42*). Because mixtures of chemicals in the environment act on alligators from these lakes, multiple points in the testosterone synthesis/hepatic inactivation pathways could be altered. A simultaneous inhibition/activation of hepatic enzymes and an alteration in the feedback mechanism to the brain could be taking place, thus explaining the altered plasma testosterone concentrations observed in animals from these lakes (*21,23*). We have also considered the possibility that the observed differences are due to genetic differences between the populations. Recent analysis has demonstrated that genetic loci that enable us to differentiate between different populations of alligators in Florida have yet to be identified, thus suggesting that genetic differences do not explain the differences in hepatic biotransformation observed in this study (*43*). Other possible explanations for the observed patterns of enzyme activity between reference and contaminated sites could include *a*) organizational differences between the sites, where androgen imprinting, similar to that reported in rats (*13*), acts on alligators, with alterations occurring in contaminated locations; *b*) activational alterations in enzyme patterns due to contaminant exposure immediately before sampling; or *c*) natural variation among populations. Further, it is important to consider the larger implications of the observed differences. Are these differences large enough to make a difference on a physiologically relevant scale? Will these differences have impacts at a population level? These questions have yet to be answered in the alligator or in other wildlife species studied to date.

References and Notes

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